IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of:

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Appln. No. 08/971,338 Group Art Unit: 1645

Filed: November 11, 1997 Examiner: M.P. Allen

FOR: GDF-1

* * *

RULE 132 DECLARATION

I, Ted Ebendal, declare and state as follows:

(1) I reside at Börjegatan 45 B, S-752 29 Uppsala, Sweden.

(2) I am a Professor and Chairman in the Department of Developmental Biology, Faculty of Medicine, Uppsala University since 1988. I hold a Ph.D. (Doctor of Philosophy) degree which was earned from Uppsala University, Sweden in 1976. A copy of my *curriculum vitae* is attached.

(3) I am an author of over 100 peer-reviewed publications in the field of neuronal growth factors and neurotrophic factors.

(4) Recombinant human GDF-1 (rhGDF-1) was provided by Michael Jarpe of Cambridge NeuroScience for use in the assays reported herein.

(5) On information and belief, rhGDF-1 was produced as follows. The cDNA of human GDF-1 (amino acid residues 255 to 373) was cloned into pRSET (Invitrogen). The construct was designed to produce a fusion protein which adds 34 amino acid residues to the N-terminus of rhDGF-1 including six histidine residues. There is an enterokinase cleavage site between the N-terminal extension and the rhGDF-1 sequence to facilitate removal of the tag. However, this extension was not removed for the assays reported herein.

(6) On information and belief, the above-described expression construct was inserted into the *E. coli* cell line BL21(DE3)pLysS to induce rhGDF-1 expression. Expression was induced by the addition of IPTG and was allowed to proceed for 4 hours. rhGDF-1 was produced in inclusion bodies.

(7) On information and belief, the inclusion bodies containing rhGDF-1 were solubilized and folded in 6 M guanidine and 100 mM dithiothreitol. Reducing agent and denaturing agent was removed by reverse phase HPLC. The protein was dried down in a Speed Vac and resuspended in 8 M urea at 5 mg/ml protein concentration. The protein solution was then diluted 1/100 to a final concentration of 50 µg/ml in refolding buffer of 10 mM reduced glutathione, 1 mM oxidized glutathione, and 50 mM Tris buffer (pH 9.0). The rhGDF-1 protein was allowed to refold for 20 hours at 25°C.

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(8) On information and belief, a sample of the refolded rhGDF-1 protein was then analyzed by reducing and non-reducing SDS-PAGE. The gel was stained with Coomassie and the proportion of dimer was determined by densitometry. The rhGDF-1 dimer was found to be approximately 20% of total protein. The rhGDF-1 preparation was stored at -80°C.

(9) The following assays were performed under my direction and the results were analyzed by me.

(10) The sample was assayed in a fibre outgrowth assay using sympathetic ganglia from embryonic day 9 chicken embryo explanted into a collagen gel. See Ebendal *et al.*, Journal of Neuroscience Research, vol. 40, pp. 276-284 for a description of the use of explanted ganglia in collagen gels. Neurotrophin-3 (NT-3) only weakly stimulates sympathetic fibre outgrowth in this assay (see panel d of Fig. 4 in Ernfors *et al.*, Proceedings of the National Academy of Science, U.S.A., vol. 87, pp. 5454-5458). Members of the TGF-beta superfamily of proteins potentiate the effects of NT-3 in this assay.

(11) The sample of GDF-1 was diluted 100-fold and then further diluted in culture medium with 1% fetal calf serum as a carrier. GDF-1 was assayed on sympathetic ganglia at a

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concentration of 2.5 to 250 ng/ml. The ganglia were examined after two days of incubation using darkfield microscopy. No fibre outgrowth was evoked by GDF-1 at any of these concentrations.

(12) Therefore, the potentiating effect of GDF-1 on neurotrophin activity could be assessed by comparing fibre outgrowth induced by NT-3 in the presence or the absence of GDF-1. Any increased fibre outgrowth caused by the combination of NT-3 and GDF-1 would be due to potentiation, instead of the effects of GDF-1 alone.

(13) The potentiating effect of GDF-1 in the sympathetic fibre outgrowth assay (Ernfors *et al.*, *id.*) was determined with human NT-3 (Austral Biologicals) at a concentration of 2 ng/ml and GDF-1 at concentrations between 0 to 250 ng/ml. Fibre outgrowth density was scored in a blinded fashion by two independent observers with culture dishes arranged in random order. Scores were recorded on a scale from 0 (no fibres) to 5 (very high density of fibres forming a circular halo around the explanted nervous tissue). The assay was repeated three times. The results below represent the mean of the scores given for each culture by each observer.

	GDF-1 concentration	Mean Score
Medium Only	0 ng/ml	0.0
NT-3 alone	0 ng/ml	1.7
GDF-1 alone	250 ng/ml	0.0
NT-3 + GDF-1	250 ng/ml	3.1
NT-3 + GDF-1	50 ng/ml	2.3
NT-3 + GDF-1	5 ng/ml	1.8

(14) The combination of GDF-1 at 250 ng/ml with NT-3 shows a significant potentiation effect in comparison to the response obtained with NT-3 alone (statistically significant difference at $P < 0.001$ using Mann-Whitney U test). There is also a clear trend of potentiation of NT-3 by GDF-1 at 50 ng/ml, although this difference is not statistically significant in the present format of the assay.

(15) The specific activity of GDF-1 in the assay shows a reasonable dose-response relationship between 50 to 250 ng/ml.

(16) In view of the above results, I conclude that GDF-1 has biological activity on neurons similar to members of the TGF-beta superfamily of proteins.

(17) I declare further that all statements made herein of my own knowledge are true and that all statements made on

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information and belief are believed true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

× Ted Ebendal
Ted Ebendal

× April 9, 1998
Date

CURRICULUM VITAE for TED EBENDAL

Born: September 21, 1948. Stockholm, Sweden
Sex: Male
Marital status: Married, 1 child (born 1974)
Address: Börjegatan 45 B, S-752 29 UPPSALA, Sweden

Education/academic degrees:

1971 Bachelor of Science. Uppsala University, Sweden
1972 Master of Science, Uppsala University
1976 Doctor of Philosophy, Uppsala University
1977 Docent in Zoology, Uppsala University
1987 Professor, Faculty of Medicine, Uppsala University

Professional Experience:

1972-76	Teaching Instructor in Zoology, Uppsala University
1977	Assistant Professor in Zoology, Uppsala University
1977	Visiting scientist at Strangeways Research Laboratory, Cambridge, England
1977-81	Docent (Associate Professor) appointment in Zoology, Uppsala University
1981-87	Research Associate Professor in Neurobiology at the Swedish Natural Science Research Council
1988-	Full Professor of Developmental Biology, Faculty of Medicine, Uppsala University

Scientific Awards: King Oscar Prize, Uppsala University (1982). Erik K. Fernström's prize to especially promising young scientists (1991).

Invited Oral Presentations have been given at about 80 international meetings.

Current research interests: Molecular and developmental neuroscience, development and repair mechanisms in the nervous system, neuronal growth factors and their receptors including NGF, NT-3, GDNF and BMPs. Homologous recombination in transgenic mice using embryonic stem cells.

Administrative and scientific duties at the Faculty of Medicine, Uppsala University Chairman at the Dept of Developmental Biology (1988-). Member of the Medical Faculty Board (1988-93). Member of various scientific priority committees etc (1988-), Vice Chairman of Neuroscience Center at Uppsala University, (1989-1995). Chairman Uppsala University Animal Research Board (1997-)

Pre-doctoral advisor: Supervised 11 PhD students of which 8 have finished their PhD thesis and three are on the way.

Post-doctoral advisor for: Wilma Friedman (USA, 1986-88), Reg Williams (Australia, 1991-94).

Organization of scientific meetings, courses etc: Organized EMBO and BMC Summer School courses for graduate students in Uppsala and at the Karolinska Institute and participated in the organization committees for international meetings in Sweden and abroad (ISDN Biennial Meeting in Tampere 1996, 5th NGF Meeting 1998).

Referee assignments etc: Exp. Cell Res., Exp. Brain Res.

Associate editor: J. Neurosci. Res., Int. J. Dev. Neurosci, Neuron, Alzheinmer's Disease.

Reviewer of applications for grants and research positions: Uppsala University, Karolinska Institute, Stockholm University, Umeå University. MRC Sweden, NSF USA and various Swedish Universities.

Member of scientific committees: The Swedish Foundation for Brain Research (1995-), The International Human Frontier Science Program (Brain Functions 1996-), The Royal Swedish Academy of Sciences National Committe on Biology (1997-).

Major funding (Principal Investigator): Swedish NFR (SEK 900,000/yr for 1997 to 1999).

Publications: Over 300 scientific papers, reviews and reports in the area of developmental neuroscience

SELECTED LIST OF PAPERS PUBLISHED BY TED EBENDAL

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Ericsson, A. 1988. Structure and expression of ß-nerve growth factor in the rat central nervous system. In: Neural Development and Regeneration (ed. A. Gorio et al.). NATO ASI Series, Vol. H 22: 245-256. Springer, Berlin.

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Rapid Communication

Glial Cell Line-Derived Neurotrophic Factor Stimulates Fiber Formation and Survival in Cultured Neurons From Peripheral Autonomic Ganglia

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Human recombinant glial cell line-derived neurotrophic factor (GDNF) was tested for its ability to stimulate fiber formation and neuron survival in primary cultures of peripheral ganglia dissected from the chicken embryo. GDNF, first characterized by its actions on central nervous system (CNS) neurons, had a marked stimulatory effect on fiber outgrowth in sympathetic and ciliary ganglia. Weaker responses were evoked in sensory spinal and nodose ganglia and in the ganglion of Remak. In addition, survival of neurons from the sympathetic and ciliary ganglia was stimulated by GDNF at 50 ng/ml. The effects were not mimicked by the distant but related protein transforming growth factor beta 1 (TGFβ1). The profile of neurons stimulated by GDNF is also distinct from the patterns of stimulation shown by nerve growth factor (NGF), stimulating strongly sympathetic but not ciliary ganglia, and ciliary neurotrophic factor (CNTF), stimulating mainly the ciliary ganglion. Moreover, using in situ hybridization histochemistry, GDNF was demonstrated to be present in the pineal gland in the newborn rat, a target organ for sympathetic innervation. The present results suggest that GDNF is likely to act upon receptors present in several autonomic and sensory neuronal populations. GDNF may serve to support fiber outgrowth and cell survival in peripheral ganglia, adding yet one more trophic factor to the list of specific proteins controlling development and maintenance of the peripheral nervous system.

Key words: trophic factor, TGFβ, chicken embryo, tissue culture, bioassay

INTRODUCTION

Development, selective survival, and function of neurons are regulated by cellular interactions mediated by a host of neurotrophic molecules. Thus, proteins with a well-characterized ability to support neurons include the family of neurotrophins [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins-3 and -4 (NT-3 and NT-4)], ciliary neurotrophic factor (CNTF), acidic and basic fibroblast growth factors (aFGF and bFGF, respectively), and insulin-like growth factors (IGF-1 and IGF-2).

A recent addition to the list of proteins with trophic effects on neurons is glial cell line-derived neurotrophic factor (GDNF) (Lin et al., 1993). GDNF was initially documented to support survival, differentiation, and high-affinity dopamine uptake in fetal dopamine neurons from the ventral mesencephalon in vitro. The active molecule was purified and partially sequenced and subsequently DNA clones encoding the novel neurotrophic factor were isolated from rat cDNA and human genomic DNA (Lin et al., 1993). Analysis of the sequences obtained showed that GDNF, a 134-amino-acid protein in its mature form, is a member of the transforming growth factor beta (TGFβ) superfamily of growth and transforming factors (see review by Massagué, 1990) but is not closely related to any of the many previously known

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members of this family (Burt, 1992; Lin et al., 1993; Burt and Law, 1994). When expressed as a recombinant protein produced in bacteria, the GDNF, after proper folding (Lin et al., 1993) had the expected dopaminotrophic activity originally described in the glial cell line from which it was derived (Schubert et al., 1974). The TGFβ proteins, the neurotrophins, and the platelet-derived growth factors A and B (PDGFs) all share some protomeric structural motifs rendering them members of the cystine-knot superfamily of growth factors (McDonald and Hendrickson, 1993). GDNF, like the other cystine-knot proteins, form homo- or heterodimers that are the biologically active entities. It is thus interesting that neurotrophic activities have been ascribed not only to NGF and the other neurotrophins, but also to PDGF and to TGFβs. Very recently, the structural characterization of human chorionic gonadotropin (hCG) has shown that it is also a member of the cysteine-knot family of proteins (Lapthorn et al., 1994) but the neurotrophic activity of hCG has not yet been reported.

So far, studies of the effects of GDNF have involved neurons of the central nervous system (CNS) such as the mesencephalic dopamine neurons and α-motoneurons (Lin et al., 1993). In addition, GDNF is expressed in the rat striatum and in other areas of the brain during development (Schaar et al., 1993; Strömberg et al., 1993). Moreover, in the adult rat brain, kainate-induced epileptic seizures induce GDNF expression in the granule cells of the adult dentate gyrus (Humpel et al., 1994), and pilocarpine-induced seizures lead to upregulation of GDNF in the striatum (Schmidt-Kastner et al., 1994).

In the present report we demonstrate that GDNF also has the ability to stimulate peripheral neurons. We document neurotrophic effects of GDNF in a series of sympathetic and parasympathetic autonomic as well as some sensory peripheral neurons in culture. It is shown that GDNF has substantial trophic effects, particularly on some autonomic neuron populations, and that these patterns of stimulation are distinct from those evoked by the neurotrophins (NGF, BDNF, NT-3, and NT-4) as well as CNTF and TGFβ1.

MATERIALS AND METHODS

Ganglia from chicken embryos at day 9 of incubation were explanted as intact ganglia for a fiber-outgrowth assay (Ebendal et al., 1978; Hedlund and Ebendal, 1978; Ebendal et al., 1980, 1984; Ebendal, 1989) or, in the case of the sympathetic paravertebral trunk ganglia and the ciliary ganglion, dissociated for a neuron-survival assay (Ebendal et al., 1985; Kullander and Ebendal, 1994). Sympathetic paravertebral ganglia and sensory spinal ganglia (dorsal root ganglia) were dissected from the lumbosacral region of the embryo. The ciliary ganglion was taken from the orbit, the nodose ganglion from the vagus nerve rostral to the heart. Finally, the ganglion of Remak was isolated from the mesorectum of the embryo (Hedlund and Ebendal, 1978). The effects of GDNF in cultures of the sensory trigeminal and spinal (dorsal root) ganglia were also tested. Ganglia or dissociated neurons were placed in collagen gels for culture as detailed by Ebendal (1989). All findings were repeated at least twice in independent experiments yielding the same results. The basic finding of a strong GDNF stimulation of the sympathetic ganlion has been repeated in more than 25 cultures established over a period of more than 6 months in our laboratory. Likewise, the survival data are based on counting of several hundred neurons on several occasions.

Recombinant human GDNF was obtained from Synergen, Inc. (Boulder, CO). The protein was produced in bacteria, refolded to yield an active molecule, and purified as a non-glycosylated disulfide-bonded homodimer as described (Lin et al., 1993). Human recombinant TGFβ1 was obtained from Boehringer Mannheim (Darmstadt, Germany). Both proteins were aliquoted to avoid repeated freeze-thawing cycles and added to the culture medium at the final concentrations indicated for each experiment. For positive effect on neuron survival, purified mouse βNGF (Ebendal et al., 1984) was added to sympathetic neurons at 5 ng/ml. To support ciliary neuron survival, an extract of the choroid coat including the pigment epithelium from the 18-day-old chicken embryo was added at a final concentration of 200 μg of total protein/ml of medium (Ebendal, 1987).

Ganglia and dissociated neurons were observed under darkfield or phase contrast optics, fiber outgrowths observed, neurons counted, and microphotographs taken after 2 days in culture.

For in situ hybridization, E17 rat fetuses were taken from pregnant Sprague-Dawley rats that had been killed by neck dislocation under deep ether anesthesia. The heads of the fetuses were rapidely frozen on dry ice. Newborn rats were killed by decapitation and the heads frozen on blocks of dry ice. Cryostat sections (14 μm thick) of the fetal and neonatal brains were cut and placed on coated slides (ProbeOn, Fisher Biotech, Orangeburg, NY). To perform in situ hybridization (Dagerlind et al., 1992), sections were thawed and hybridized with two antisense oligonucleotide probes (both 50-meres, positions corresponding to nucleotides 456–505 and 540–589, respectively, in the sequence deposited under GenBank accession number L15305; see Lin et al., 1993). These two oligonucleotide probes had no similarities to sequences deposited in GenBank and generated identical in situ hybridization patterns in tissue. As a negative control, a random probe of the same length and GC content was applied to adjacent sections. This

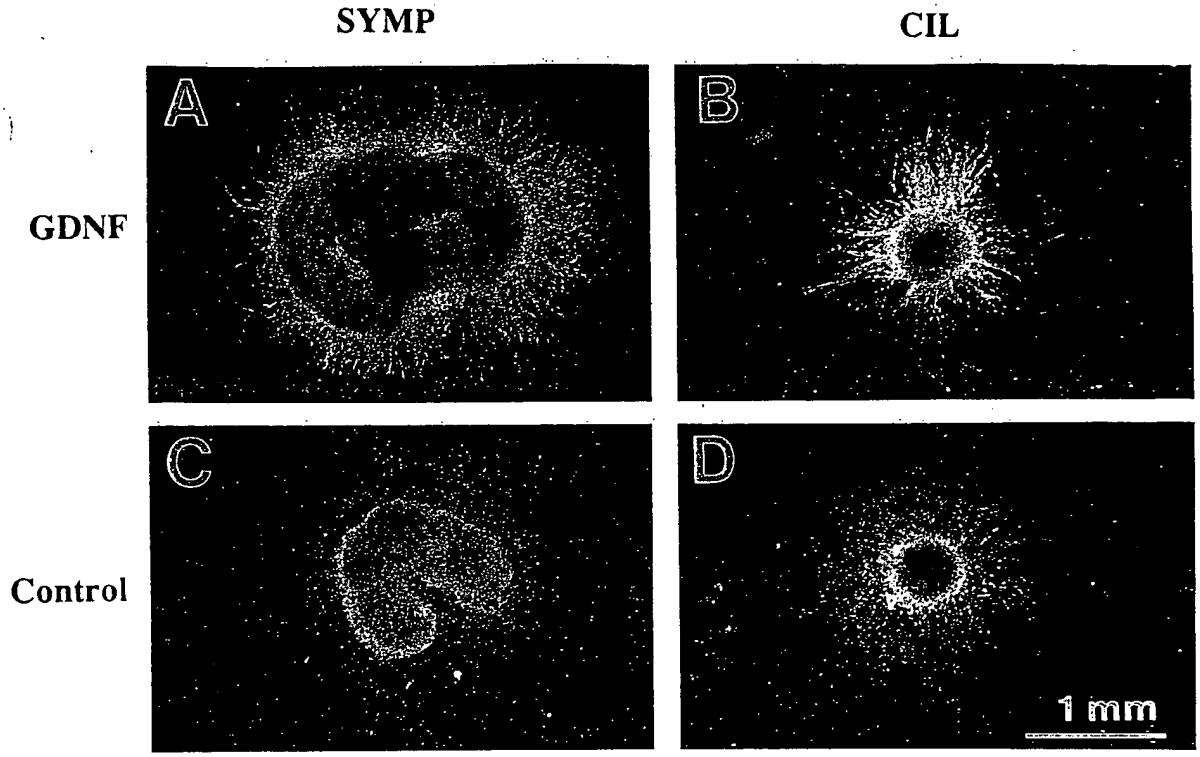

Fig. 1. The response to GDNF in explanted sympathetic and ciliary ganglia. **A:** A sympathetic ganglion cultured for 2 days in the presence of GDNF at 100 ng/ml. A dense, short fiber outgrowth is evident around the ganglion. **B:** The same conditions as in A but with a ciliary ganglion. The formation of fiber fascicles is evident. **C:** A negative control with a sympathetic ganglion cultured for 2 days without the support of GDNF. **D:** A ciliary ganglion in control medium. Darkfield micrographs of living cultures.

random probe did not give rise to any signals above background in examined tissues. The oligoprobes were end labeled with ^{35}S-dATP using terminal deoxyribonucleotidyl transferase. The probes were then purified (Nensorb columns) and applied to the tissue sections at 42°C overnight in a hybridization solution. Following this, the slides were rinsed in 1× SSC at 54°C, dehydrated in a series of ethanols, and air-dried. The slides were dipped in Kodak NTB-2 film emulsion and exposed for 6–8 weeks at −20°C, developed in a photographic developer, fixed, lightly stained with cresyl violet, and mounted. The sections were observed and photographed with dark- and brightfield illumination (Nikon Microphot FX microscope).

RESULTS

GDNF, present at a concentration of 50–100 ng/ml, was consistently found to evoke marked fiber outgrowth in the explanted sympathetic and ciliary ganglia cultured for 2 days (Fig. 1A,B). The outgrowth consisted of dense, short fibers around the sympathetic ganglia. From the ciliary ganglion, GDNF evoked the formation of fairly thick fiber fascicles. The concentrations tested were 0.5, 5, 50, 100, 200, 400, and 1,000 ng/ml of GDNF in the medium and optimum fiber responses were obtained in the sympathetic ganglia with 50 and 100 ng/ml. The resulting outgrowth in the sympathetic ganglion was less prominent than that evoked by NGF at 3–5 ng/ml (Ebendal, 1989), but the fibers were dense and markedly tufted. For comparison, representative NGF-induced outgrowth responses in sympathetic ganglia in this assay can be found in a recent report by Kullander and Ebendal (1994).

The ciliary ganglion responded by fiber outgrowth to GDNF in the range of 5–1,000 ng/ml, thus at a wider range of concentrations than found effective to evoke fiber outgrowth in the sympathetic ganglion. Control cultures of sympathetic and ciliary ganglia without added GDNF were totally devoid of fiber outgrowth (Fig. 1C,D).

Some effects of GDNF on fiber outgrowth were

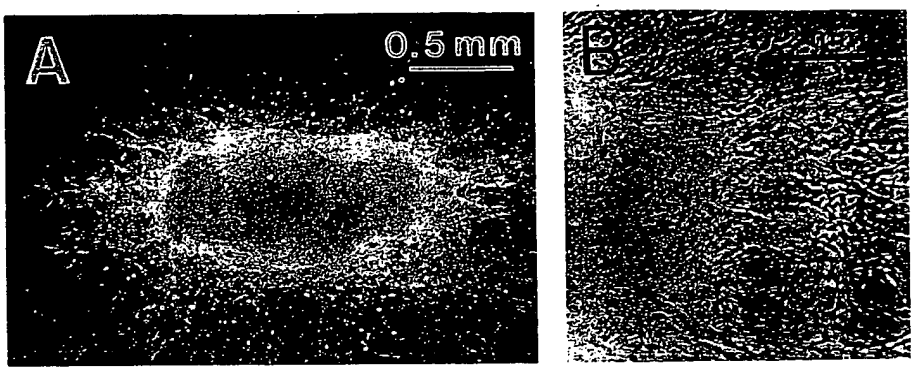

Fig. 2. Effects of GDNF on fiber outgrowth in the nodose ganglion. Some thick fiber fascicles have formed from this sensory ganglion cultured for 2 days with 100 ng/ml of GDNF. **A:** Darkfield overview of the ganglionic explant. **B:** Phase contrast close-up showing the heavily fasciculated fibers induced by GDNF.

also observed in the nodose ganglion (Fig. 2A). A few thick fiber fascicles (Fig. 2B) were formed from this sensory ganglion in response to 100 ng/ml of GDNF over the 2 day culture period. It should be stressed that the outgrowth response was markedly weaker than that evoked by NT-3 at 5–10 ng/ml (for a comparison, see the results obtained in this assay presented by Ernfors et al., 1990) in this epidermal placode-derived ganglion. GDNF also weakly stimulated some fiber outgrowth in the spinal dorsal root ganglia (not shown) and in the trigeminal ganglion (data not shown). The effect of GDNF on fiber outgrowth from Remak's ganglion (data not shown) was only marginal at 50 ng/ml, in contrast to the very strong fiber outgrowth evoked in this ganglion by NT-3 at concentrations of 3–5 ng/ml (Ernfors et al., 1990; Kullander and Ebendal, 1994).

The specificity of the GDNF stimulation in the ganglia was tested by comparisons of neurotrophic responses evoked by TGFβ1. Sympathetic ganglia cultured for 2 days with TGFβ1 at 10 and 100 ng/ml were found to lack fiber outgrowth and thus resemble the control explants in ordinary culture medium (Fig. 3A,B), in contrast to the dense fiber outgrowth formed in response to GDNF at 100 ng/ml (Fig. 3C,D). The specificity of the GDNF stimulation was tested also in the ciliary ganglion. In control medium no formation of fibers occurred (Fig. 4A). The ciliary ganglion likewise showed no response to TGFβ1 at 10 or 100 ng/ml (Fig. 4B) present for 2 days in the culture. In contrast, GDNF present at 100 ng/ml resulted in the formation of dense fiber fascicles (Fig. 4C).

In addition to selective stimulation of fiber outgrowth in some peripheral ganglia, GDNF has survival effects on dissociated ganglionic neurons in culture. A dose-response relationship between survival of sympathetic neurons and the presence of GDNF in the concentration range 1–100 ng/ml is shown in Figure 5A. Half-maximum survival effect was seen at about 50 ng/ml GDNF and represents rescue of nearly half of the seeded neurons. Increasing the dose of GDNF above 100 ng/ml did not further enhance survival rate (data not shown). A similar response was found in the dissociated ciliary neurons stimulated by GDNF (Fig. 5B). For sympathetic neurons, NGF at 5 ng/ml will rescue close to 100% of the neurons. The same is true for ciliary neurons when grown with a choroid extract from the eye of the embryonic day 18 chicken (Ebendal, 1987).

In order to examine the presence of GDNF mRNA in target tissue for peripheral autonomic innervation, we used the rat since the chicken GDNF sequence is not yet known. In situ hybridization using two non-overlapping GDNF specific oligonucleotide probes revealed a strong signal in the pineal gland of the newborn rat (Fig. 6A). The signal appeared to be present over most but not all pinealocytes (Fig. 6B). A similar signal was seen also in the pineal gland of the fetal rat at embryonic day 17 (not shown).

DISCUSSION

In the present report we show that GDNF has a pattern of stimulating ganglionic neuron populations distinct from those described earlier for the neurotrophins NGF, BDNF, NT-3, and NT-4, as well as from that of the ciliary neurotrophic factor CNTF. Thus, the Remak ganglion is strongly stimulated by NT-3 (Ernfors et al., 1990; Kullander and Ebendal, 1994) due to its abundant expression of the tyrosine kinase receptor TrkC (Williams et al., 1993), whereas GDNF had very limited stimulatory action on this ganglion. The sympathetic ganglion was markedly stimulated by GDNF but not to the extent of that seen with NGF. The response involved

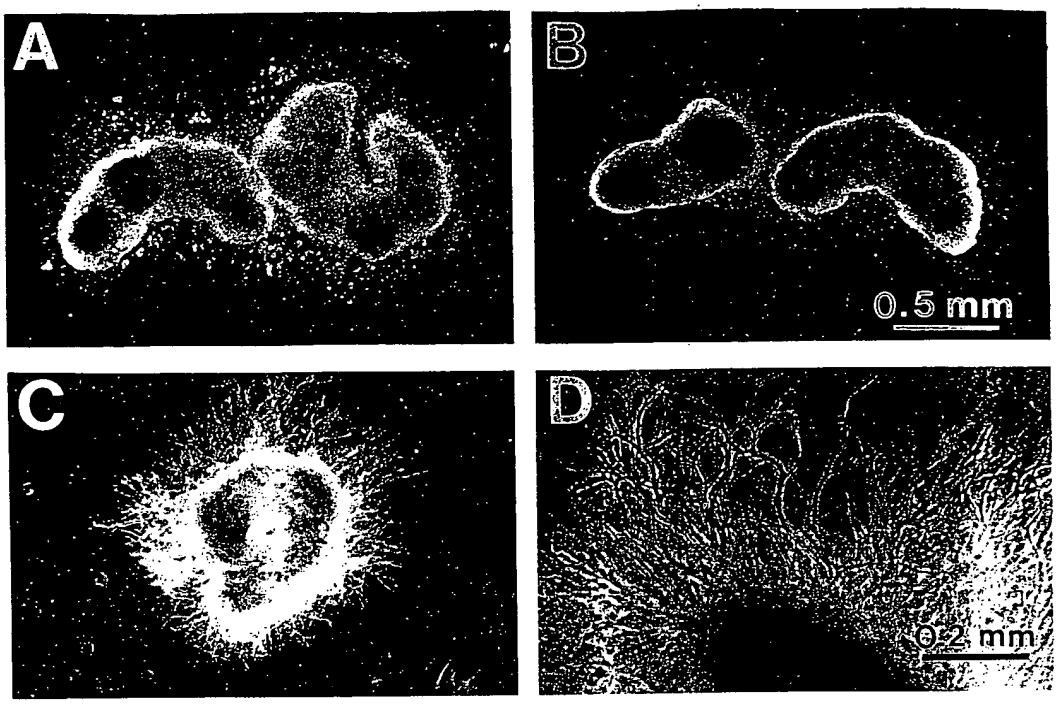

Fig. 3. Specificity of the GDNF stimulation in the sympathetic ganglion. **A:** Sympathetic ganglion in control medium after 2 days of culture. **B:** A sympathetic ganglion cultured for 2 days with TGFβ1 at 100 ng/ml. **C:** Sympathetic ganglion with GDNF at 100 ng/ml present in the medium for the culture period of 2 days. Only with GDNF present are fibers formed. Darkfield microscopy. **D:** Phase contrast detail of fiber outgrowth from a sympathetic ganglion grown for 2 days with GDNF at 100 ng/ml.

both a survival and a fiber outgrowth response. On the other hand, GDNF also stimulated the ciliary ganglion which NGF does not. It remains to be examined whether there are time-dependent switches in the responsiveness to GDNF in the autonomic and sensory neurons examined here in analogy to changing dependency for different neurotrophins found earlier in sensory neurons (Buchmann and Davies, 1993). This could provide insight into potential temporal windows during development when these peripheral neurons depend on GDNF. In situ hybridization has been utilized to study GDNF expression and its relationship to innervation. It has been shown that there is an early expression of GDNF in the brain which is later downregulated (Schaar et al., 1993; Strömberg et al., 1993), and such an expression pattern may argue for developmental regulation of GDNF to serve neurosupportive functions during restricted periods of neurodevelopment.

Embryonic tissue extracts and explants have, in a number of cases, been shown to stimulate ganglionic neurons in culture (Ebendal et al., 1980, 1984, 1985; Ebendal, 1987). Many of these effects have been ascribed to the presence of CNTF, but also to aFGF and bFGF. GDNF is one more factor which may have contributed to these observed stimulatory effects.

In order to examine if GDNF mRNA is present in any target areas for peripheral innervation we used the perinatal rat, since the GDNF gene has not yet been sequenced from the chicken. In view of the marked effects of GDNF on the chicken sympathetic ganglia, we chose to study one target organ for the sympathetic superior cervical ganglion, i.e., the pineal gland. Consistent with the in vitro data, the pineal gland was found to express relatively high levels of GDNF mRNA during late prenatal and early postnatal stages of development (Fig. 6). Thus, GDNF may be one of the target-derived trophic factors stimulating the sympathetic nerves that innervate targets such as the pineal gland.

In the present study, specific activities of GDNF on the responsive peripheral neurons were found in the range of 50–100 ng/ml of the factor. This contrasts with the lower concentrations found to evoke tyrosine hydroxylase (TH) induction in cultured ventral mesencephalic neurons. Thus, Lin et al. (1993) found GDNF to be

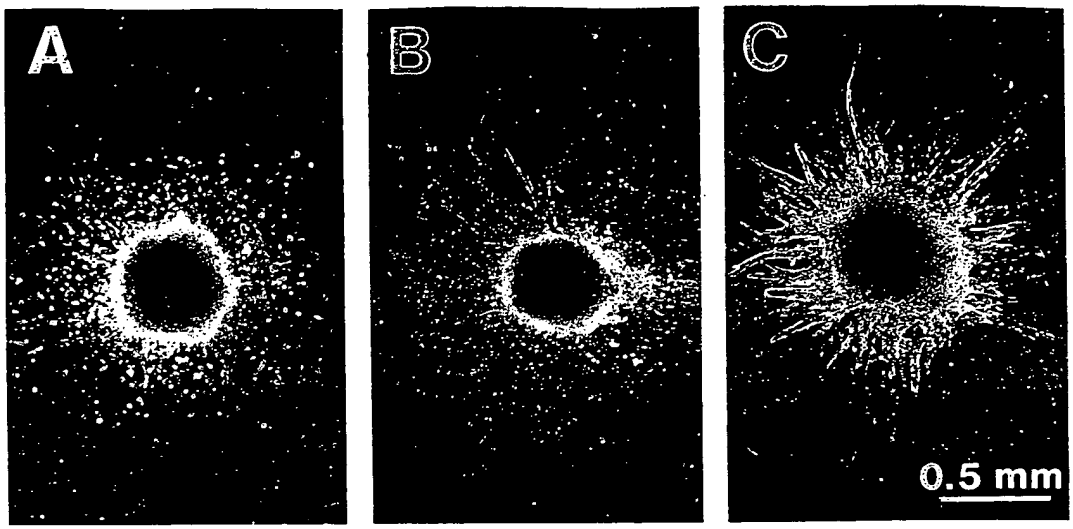

Fig. 4. Specificity of the GDNF stimulation in the ciliary ganglion. **A:** Ganglion grown in control medium for 2 days. No formation of fibers occurs. **B:** A ciliary ganglion showing no response to TGFβ1 at 100 ng/ml. **C:** A ciliary ganglion cultured for 2 days with GDNF present at 100 ng/ml. GDNF, but not TGFβ1, elicited dense fiber fascicles. Darkfield microscopy.

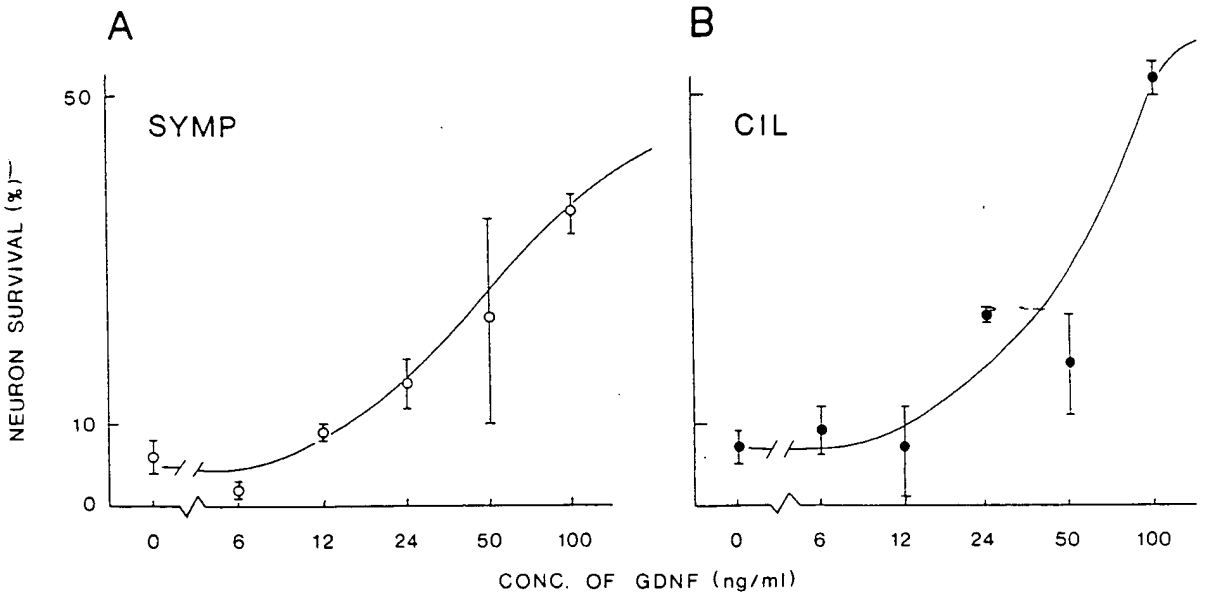

Fig. 5. Survival effect of GDNF on dissociated ganglionic neurons in culture. **A:** Dose-response curve for survival of sympathetic neurons in response to GDNF. **B:** The same experiment shown for ciliary ganglionic neurons. Each graph is based on two independent experiments, with each value based on observations of the survival in several hundred neurons. Mean value and range are shown. Survival is shown relative to the number of initially seeded neurons.

effective at a concentration of 0.04–1 ng/ml, which is in line with what is generally expected for a high-affinity interaction between a growth factor and its specific receptor. It is therefore possible that the present effects are the result of GDNF mimicking a related endogenous growth factor that might act on the peripheral neurons at higher efficiency.

The present experiments showed that GDNF stim-

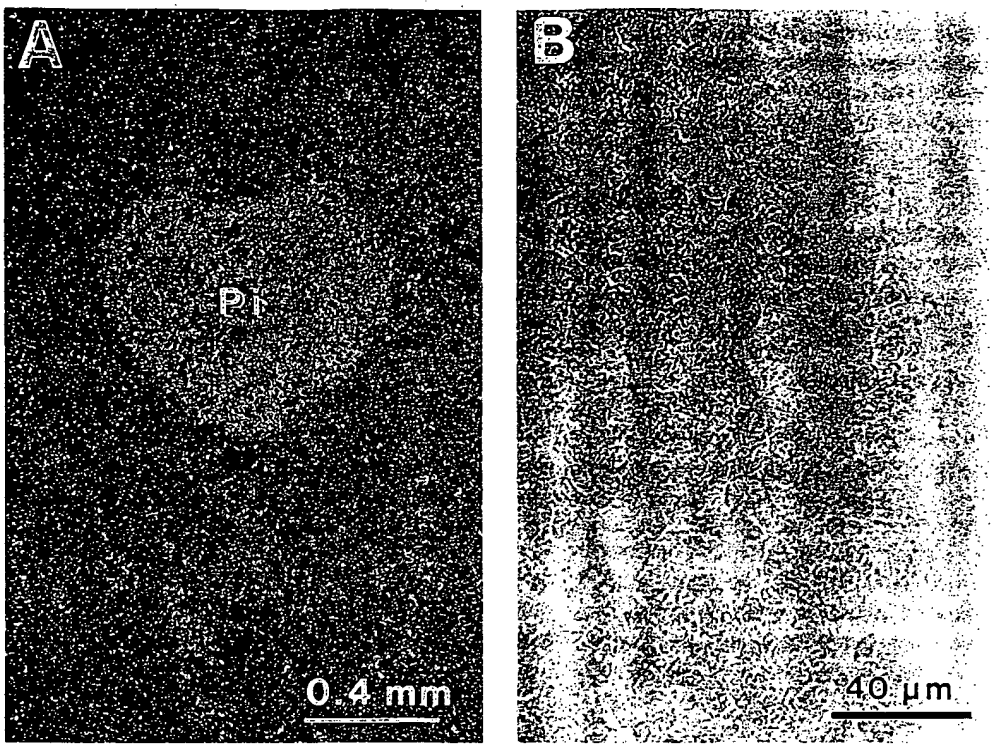

Fig. 6. In situ hybridization with an oligonucleotide probe complementary to rat GDNF mRNA. **A:** Darkfield overview of the pineal gland (Pi) of the newborn rat. **B:** Brightfield micrograph showing hybridization signal over many but not all pinealocytes.

ulated the ciliary ganglion to form nerve fibers in a concentration range of 5–1,000 ng/ml, thus at a wider range of concentrations than was effective for the sympathetic ganglion. This may reflect differences in the growth properties of the ganglia when taken to culture and not necessarily point to different receptor mechanisms. We have earlier found that NT-3 marginally stimulates fiber formation in the ciliary ganglion (Ernfors et al., 1990). The response to GDNF is stronger than that elicited by NT-3 and also appears stronger than the fiber outgrowth response evoked by recombinant CNTF in explanted ciliary ganglia (see Carri et al., 1994).

Before the cloning of GDNF, members of the TGFβ superfamily of growth factors have been considered to possess various neurotrophic and neuron differentiating properties. Thus TGFβ1 promotes the survival of motoneurons in culture, but fails to support the survival of dissociated sympathetic neurons (Martinou et al., 1990), in contrast to the action of GDNF shown here. Activin has been found to stimulate survival in some populations of neurons including the chicken embryo retina (Schubert et al., 1990), but has failed to support the survival of dissociated ciliary ganglionic neurons in an assay similar to the one used here. Again, this contrasts with the supportive effect of GDNF on dissociated ciliary ganglion neurons in the present report.

TGFβ1, -2, and -3 have all been localized in the nervous system using specific peptide antibodies (Flanders et al., 1991). TGFβ1 was mainly found in the meninges, whereas TGFβ2 and TGFβ3 were found in neurons and radial glia. It was also found that TGFβ2 and -3 inhibited the survival of ciliary ganglion neurons in the presence of an eye extract normally supporting these neurons in dissociated cultures (Flanders et al., 1991). This could again indicate the presence of receptors for TGFβs being present on these primary neurons. Supportive of this, TGFβ1 and -2 were found to enhance sensory dorsal root ganglion neuron survival and result in increased levels of substance P (Chalazonitis et al., 1992), actions suggested to be exerted synergistically with NGF. Other additive or synergistic effects of GDNF with the neurotrophins are thus possible, but have yet to be studied.

The present data suggest that a receptor mechanism for GDNF or a related trophic factor is present in developing sympathetic and ciliary neurons and that the receptor specificity is such that GDNF is an agonist ligand whereas TGFβ1 fails to elicit the signaling events lead-

ing to fiber outgrowth or neuron survival in these neurons. Several receptors for the members of the TGFβ family have been identified (see reviews by Massagué, 1992; Lin and Lodish, 1993), but the GDNF receptor, or receptors, remains uncharacterized. It seems likely that the receptor molecules for GDNF are related to the other signaling receptors mediating the actions of the members of the TGFβ superfamily. Activin receptors (Matthews and Vale, 1991; Attisano et al., 1992) and a TGFβ receptor serine/threonine kinase receptor have been cloned (Lin et al., 1992) and classified as type II transmembrane receptors of approximately 75 kDa. Also several type I receptors have been cloned (ten Dijke et al., 1994). It is considered that TGFβ actions are mediated via signal transduction involving the heterodimerization of receptors of the two types and that type II receptors exhibit a higher degree of specificity for their cognate ligands than the type I receptors (ten Dijke et al., 1994). It has recently been shown that the receptors of class II bind the TGFβ and as a second step recruit receptors of type I, which as a result is phosphorylated on serines and threonines as an initial step in a cascade of signaling phosphorylation of downstream proteins (Wrana et al., 1994). Recently, a chicken type II TGFβ receptor has been cloned (Barnett et al., 1994) and has considerable sequence similarity to the mammalian TGFβ receptor II in the kinase domain but is highly divergent in the N-terminal ligand binding part of the receptor. Whether this receptor is mediating the GDNF effects seen presently in peripheral chicken neurons remains to be studied.

Since TGFβ induces the formation of extracellular matrices (Massagué, 1990; Lin and Lodish, 1993) similar effects may be evoked by GDNF. Such a mechanism might be particularly important in the development of the peripheral nervous system where growing axons come into contact with a rich extracellular matrix. It has also been shown that various molecular components of such matrices enhance the formation of fiber outgrowth from the ganglia studied here (Carri et al., 1988). A further possible mechanistic link between GDNF and the extracellular matrix involves the possibility that such matrices bind locally secreted GDNF to enhance nerve growth and neuron differentiation, as has been shown for other members of the TGFβ superfamily (Massagué, 1990; Lin and Lodish, 1993).

The importance of GDNF for sympathetic ganglion development in vivo remains unclear. Null mutations of members of the neurotrophin family in mice suggest that GDNF does not have a function that is redundant to that of the neurotrophins. Thus the superior cervical ganglion will lose neurons in the mice homozygous for the null mutation. However, the temporal relationships have not been defined so the contribution of GDNF to survival of peripheral neurons cannot yet be established. Homozygous NGF null mutant mice (Crowley et al., 1994) show marked reductions in the sympathetic superior cervical ganglion, with up to 80% reduction of the ganglionic volume, losses of neurons, and the presence of abundant pycnotic nuclei 3 days after birth. Moreover, inactivation of the NT-3 gene in mice (Ernfors et al., 1994; Fariñas et al., 1994) leads to a 50% loss in neurons in the superior cervical ganglion. Thus, the normal expression of GDNF is not sufficient to maintain the sympathetic neurons in animals lacking NGF or NT-3 during development. It is possible that the small remaining population of neurons may represent GDNF-dependent cells or that GDNF affects a wider population of NGF- and NT-3-dependent neurons during other time windows of development (cf. Buchmann and Davies, 1993).

These complexities notwithstanding, we have demonstrated that GDNF has a stimulatory effect on sympathetic and ciliary ganglionic neurons. The data suggest the possibility that GDNF, or an endogenous ligand similar to GDNF, has potent neurotrophic functions supporting cell survival and neurite promotion in these neurons during stages of their development, with a spectrum of responsive neurons not shared by other known trophic factors. The present report also shows that sympathetic ganglion explants offer a robust and simple way to assay for the activity of GDNF, and possibly also for related molecules.

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Biochemistry

Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: Developmental and topographical expression in the brain

(nerve growth factor family/cDNA/neurotrophic factor/hippocampal neurons/nerve growth factor receptor binding)

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ABSTRACT **We have used a pool of degenerate oligonucleotides representing all possible codons in regions of homology between brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) to prime rat hippocampal cDNAs in the polymerase chain reaction. The amplified DNA included a product with significant similarity to NGF and BDNF, which was used to isolate a 1020-nucleotide-long cDNA from a rat hippocampal library. From the nucleotide sequence, a 282-amino-acid-long protein with ≈45% amino acid similarity to both pig BDNF and rat NGF was deduced. In the adult brain, the mRNA for this protein was predominantly expressed in hippocampus, where it was confined to a subset of pyramidal and granular neurons. The developmental expression in brain showed a clear peak shortly after birth, 1 and 2 weeks earlier than maximal expression of BDNF and NGF, respectively. It was also expressed in several peripheral tissues with the highest level in kidney. The protein, transiently expressed in COS cells, was tested on chicken embryonic neurons and readily stimulated fiber outgrowth from explanted Remak's ganglion and, to a lesser extent, the nodose ganglion. A weak, but consistent, fiber outgrowth response was also seen in the ciliary ganglion and in paravertebral sympathetic ganglia. Moreover, the protein displaced binding of NGF to its receptor, suggesting that it can interact with the NGF receptor. Thus, this factor, although structurally and functionally related to NGF and BDNF, has unique biological activities and represents a member of a family of neurotrophic factors that may cooperate to support the development and maintenance of the vertebrate nervous system.**

During development of the vertebrate nervous system, a vast overproduction of neurons is compensated for by naturally occurring neuronal death, which is regulated by their targets (1). Within the targets, specific proteins, referred to as neurotrophic factors, are produced in limiting amounts and the release of these proteins is believed to regulate both the timing and the extent of innervation (2).

In the peripheral nervous system, the most well-characterized neurotrophic factor, nerve growth factor (NGF), supports the development of sympathetic and neural crest-derived sensory neurons, and in the adult the maintenance of the sympathetic nervous system is critically dependent on NGF (3, 4). In agreement with a trophic role of NGF for adult sympathetic neurons, the levels of both NGF mRNA and protein correlate with the density of sympathetic innervation (5, 6). NGF mRNA and protein have also been found in the brain, with the highest levels in hippocampus and cerebral cortex, to which the major cholinergic pathways in the brain project (7–10). Basal forebrain cholinergic neurons can be prevented from dying after axonal transection by addition of NGF (11–15) and they respond to NGF *in vivo* by a marked increase in fiber outgrowth (16).

In addition to NGF, one other protein, termed brain-derived neurotrophic factor (BDNF), has been shown to be present in low amounts (17), secreted from cells (18), and to support survival of embryonic sensory neurons *in vivo* (19). In common with NGF, BDNF supports the survival of neural crest-derived embryonic sensory neurons *in vitro*, but nonoverlapping trophic activities are suggested by the finding that BDNF also supports placode-derived neurons from the nodose ganglia and retinal ganglion cells (20, 21), which are less sensitive to NGF (22, 23). Regulation of neuronal survival *in vivo* in the brain by BDNF has not yet been demonstrated, although its sites of synthesis have recently been mapped by *in situ* hybridization where a high level of labeling was found in hippocampal neurons (24).

NGF is synthesized as a preproprotein and the structure of both the precursor and the mature protein has been deduced from cDNA and genomic clones (25, 26). More recently, a genomic clone has been isolated for porcine BDNF (18). Of considerable interest is the finding that the mature BDNF and NGF proteins show striking amino acid similarities, suggesting that they are structurally related and may be members of a family of neurotrophic factors (18).

In this study, we report on the cloning and expression of an additional member of the NGF family.¶ Due to its restricted expression in the brain, being mostly confined to a subset of pyramidal and granular neurons in the hippocampus, we have named this protein hippocampus-derived neurotrophic factor (HDNF).

MATERIALS AND METHODS

RNA Preparation, Molecular Cloning, and DNA Sequencing. Polyadenylylated RNA [poly(A)$^+$] was prepared as described (27). For cloning, rat hippocampus poly(A)$^+$ RNA (5 μg) was used as a template for synthesis of single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase (Pharmacia). Six separate mixtures of 28-mer oligonucleotides representing all possible codons corresponding to the amino acid sequence KQYFYET (5′-oligonucleotide) and WRFIRID (3′-oligonucleotide) were synthesized on an Applied Biosystems A381 DNA synthesizer. The 5′-oligonucleotide contained a synthetic *Eco*RI site and the 3′-oligonucleotide contained a synthetic *Hin*dIII site. Each mixture of oligonucleotides was then used to prime the amplification of hippocampal cDNA (25 ng) by the polymer-

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; HDNF, hippocampus-derived neurotrophic factor; PCR, polymerase chain reaction.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34643).

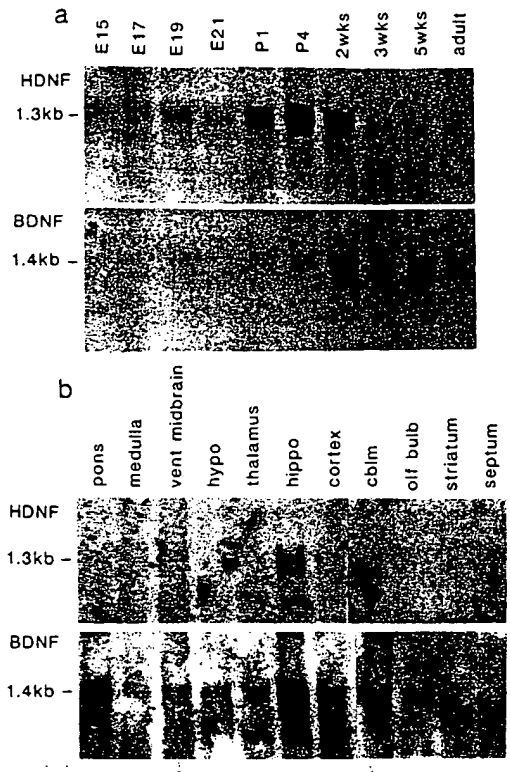

FIG. 2. Developmental and regional expression of HDNF and BDNF mRNA in rat brain. (*a*) Poly(A)$^+$ RNA (20 μg per slot) isolated from Sprague–Dawley rat brain at the indicated developmental stages was hybridized to the indicated probes (HDNF and BDNF). Adult rats were 12 weeks old. E, embryonic day; P, postnatal day; wks, weeks. (*b*) Same analysis as in *a* using poly(A)$^+$ RNA (20 μg per slot) isolated from the indicated regions of adult male Sprague–Dawley rat brain. Medulla, medulla oblongata; hypo, hypothalamus; hippo, hippocampus; cortex, cerebral cortex; cblm, cerebellum; olf, olfactory bulb.

brain showed remarkable regional specificity with high levels in hippocampus compared with other brain regions analyzed (Fig. 2*b*). In fact, cerebellum was the only other region where HDNF mRNA was clearly detected, with the exception of cerebral cortex, which showed a weak signal. BDNF mRNA was more widely distributed in rat brain, although hippocampus also contained the highest amount, followed by cerebral cortex, pons, and cerebellum (Fig. 2*b*).

Neurons Expressing HDNF and BDNF mRNA Are Located in a Distinct Topographical Arrangement in Hippocampus. Anterior sections of the dorsal hippocampus showed neurons expressing high levels of HDNF mRNA primarily confined to the medial part of CA1 and CA2 (Fig. 3 *a* and *c*). Few HDNF mRNA-expressing neurons were also found in lateral parts of CA1. Granular cells of the dentate gyrus were also highly labeled (Fig. 3*a*). CA3 and hilar cells of the dentate gyrus showed no labeling for HDNF mRNA at any level (Fig. 3*d*). No labeling was seen over any sections after hybridization to a control probe, complementary to the specific HDNF probe. Adjacent sections hybridized to a BDNF-specific probe revealed labeling over granular neurons in the dentate gyrus (Fig. 3*b*), although possibly with lower intensity than that seen after hybridization for HDNF mRNA. Strong labeling with the BDNF-specific probe was found over neurons in the hilar region (Fig. 3*e*), CA3, and part of CA2 (Fig. 3*b*). Few BDNF mRNA-expressing neurons, which appeared to be less intensively labeled, were also detected in CA1 and CA2 (Fig. 3*b*). Intensely labeled neurons were seen in claustrum, located lateral to the external capsule. This region showed no labeling for HDNF mRNA.

Neurotrophic Activities of HDNF in Explanted Chicken Embryonic Ganglia. The 1020-bp HDNF cDNA insert was cloned in the expression vector pXM (34), designed for transient expression in COS cells. Two plasmid constructs were isolated, containing the HDNF insert either in the correct or opposite orientation for translation of the HDNF protein. The latter construct was used as a negative control. Included was also a construct containing the rat NGF gene (36). The different constructs were transfected into COS cells and 3 days later conditioned medium was tested for biological activity in bioassays that measured fiber outgrowth from various chicken embryo ganglia. A marked stimulation of neurite outgrowth, consistently resulting in circular or oval fiber halos, was seen in the ganglion of Remak, a ganglionated nerve trunk in the mesorectum of the chicken embryo (38, 39) (Fig. 4*a*). Although NGF is known to stimulate the explanted ganglion of Remak (39), it was far less efficient than HDNF (Fig. 4*b*). A modest stimulation of fiber outgrowth was also seen with HDNF in the nodose ganglion, consisting of neurons exclusively derived from an epidermal placode (22)

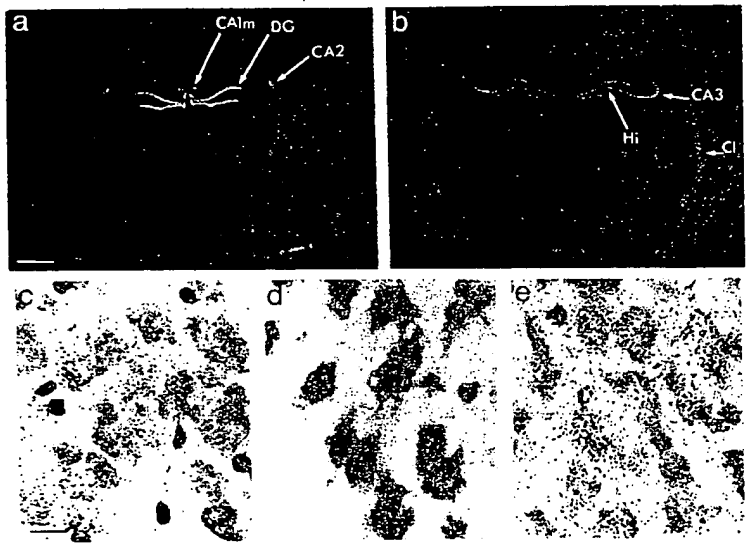

FIG. 3. Expression of HDNF and BDNF mRNA in hippocampal neurons. Rat (Sprague–Dawley) brain sections hybridized to either HDNF- or BDNF-specific oligonucleotide probes. (*a*) Autoradiogram from a section at the level of hippocampus hybridized to the HDNF-specific probe. Note labeling over medial CA1, CA2, and the dentate gyrus. (*b*) Adjacent section hybridized to a BDNF-specific probe. Note labeling over CA2 and CA3 as well as hilar cells and dentate granule layer. (*c*) Pyramidal neurons in medial CA1 labeled with the HDNF-specific probe. (*d*) Nonlabeled hilar neurons after hybridization to the HDNF-specific probe. (*e*) Hilar neurons labeled with the BDNF-specific probe. DG, dentate gyrus; CA1m, CA1 medial; Hi, hilus of dentate gyrus; Cl, claustrum. (*a* and *b*, bar = 1.3 mm; *c–e*, bar = 10 μm.)

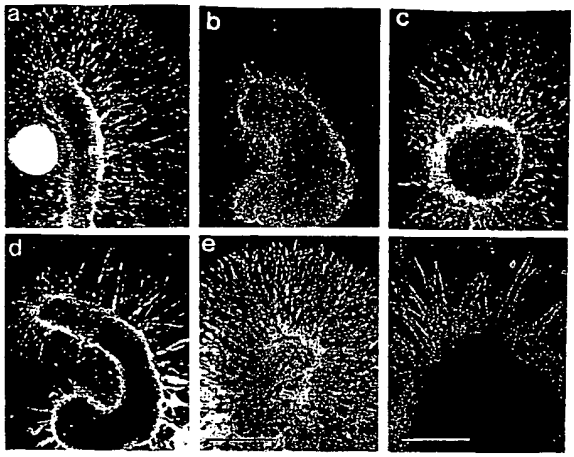

FIG. 4. Stimulation of fiber outgrowth from chicken embryonic ganglia. Biological activity of recombinant HDNF shown as effects on different nerve tissues from the chicken embryo. Remak ganglion stimulated by HDNF (*a*) or NGF (*b*). (*c*) Nodose ganglion with HDNF. Paravertebral sympathetic ganglion in response to HDNF (*d*) and recombinant rat NGF (*e*). (*f*) Ciliary ganglion with HDNF. All figures show ganglia after 1.5 days in culture. Dark-field microscopy. (Bars = 0.3 mm.)

(Fig. 4*c*). Again, HDNF was superior to NGF in evoking this response. A weak, but consistent, fiber outgrowth response with HDNF was seen in paravertebral sympathetic trunk ganglia (Fig. 4*d*), which, however, was much less pronounced compared with the massive response to rat NGF (Fig. 4*e*). In the ciliary ganglion, a weak but consistent fiber outgrowth response, manifested by the projection of short neurite fascicles, was seen with HDNF but never with NGF (Fig. 4*c*). In the dorsal root ganglia, HDNF stimulated neurite outg[illegible]h to the same extent as NGF.

Displacement of NGF Binding to PC12 Cells by HDNF. Concentrated conditioned medium from transfected COS cells was tested for its ability to compete for binding ^{125}I-labeled NGF (^{125}I-NGF) to its receptor on PC12 cells. The concentration of ^{125}I-NGF used allowed ≈80% of the labeled NGF to be bound to the low-affinity receptor site in the absence of competition (40). Twenty-five times concentrated medium containing the HDNF protein displaced ≈70% of the labeled NGF and a 20% displacement was seen after a 25-fold dilution (Fig. 5). In contrast, 25 times concentrated medium from COS cells transfected with the HDNF cDNA in the opposite orientation did not show any displacement. Concentrated medium from cells transfected in parallel with a rat NGF gene displaced 50% of the labeled NGF when diluted 250 times.

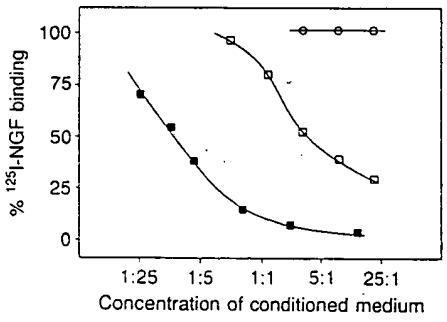

FIG. 5. Displacement of ^{125}I-NGF from its receptor on PC12 cells by HDNF and NGF. Serial dilutions of transfected COS cell medium with (□) or without (○) HDNF or containing rat NGF (■) were [illegible]ed for their ability to displace ^{125}I-NGF from its receptor on [illegible] cells. Data are from two independent experiments that showed a variation of ±20%.

DISCUSSION

The cDNA clone isolated in this study encodes a protein, HDNF, with a remarkable sequence similarity to both NGF and BDNF and therefore represents an additional member of a family of neurotrophic proteins. Recently (at the time of submission of this manuscript), two groups (41, 42) independent of us isolated genomic clones for a protein (neurotrophin 3) from mouse and rat, respectively, which is identical to the neurotrophic protein characterized in this study. Our cDNA clone predicts a 282-amino-acid-long protein, which is 24 amino acids longer than the protein deduced from the genomic clones (41, 42). Two alternative start sites for translation of the NGF protein have been proposed; the first is located in a separate 5′ exon (43). The second start site, located in the 3′ exon, is also efficiently used for translation of the NGF protein (36, 44) and generates a 68-amino acid shorter protein. Thus, the structure of our cDNA clone indicates that the HDNF protein utilizes two alternative start sites for translation, located in separate exons, and suggests that the genomic organization of HDNF and NGF is very similar.

In peripheral ganglia bioassays, HDNF showed neurotrophic activities that were to some extent reminiscent of both NGF and BDNF. Thus, in similarity to BDNF (20), HDNF stimulated fiber outgrowth from the nodose ganglia and, as for NGF, evoked a fiber outgrowth response in sympathetic ganglia. In the latter case, however, the response was clearly weaker than with NGF. The partially overlapping activities seen *in vitro* may reflect a cooperation of these factors *in vivo*, where two or more proteins from the same family may support the development and/or maintenance of specific neurons. The most striking stimulation of fiber outgrowth evoked by HDNF was seen in the peripheral, autonomic, ganglion of Remak containing mostly cholinergic but also some adrenergic neurons (38, 39). This effect was clearly more pronounced than effects seen with NGF (39), suggesting that HDNF also evokes trophic responses different from both NGF and BDNF. In agreement with this, HDNF showed a weak, but consistent, neurite outgrowth response in the ciliary ganglion, which does not respond to NGF or BDNF. The ciliary ganglion is known to respond to ciliary neurotrophic factor (45), which lacks a signal sequence, but could be released by an as yet unknown mechanism (46). Thus, HDNF is the only secreted neurotrophic factor today that is known to affect fiber outgrowth, at least *in vitro*, from the ciliary ganglion.

The HDNF protein displaced ^{125}I-NGF from PC12 cells, indicating that it can interact with the NGF receptor. With the assumption that NGF and HDNF were produced in equal amounts in parallel transfections and that the conditioned medium lacks interfering substances, the interaction of NGF to its receptor was 30-fold more efficient. PC12 cells have both low- and high-affinity receptors but only the high-affinity receptor mediates a biological response (47). The fact that recombinant rat NGF readily stimulated neurite outgrowth from PC12 cells, whereas HDNF, even at 30-fold higher concentrations than NGF, did not suggests that HDNF can only interact with the NGF receptor in its low-affinity form. It therefore appears likely that the biological responses elicited by HDNF are mediated by either a separate second messenger system compared with NGF or that the HDNF receptor is different from the NGF receptor.

In similarity with NGF, HDNF mRNA was found in several peripheral rat tissues, with the highest level in kidney. Hybridization of the same filters to a rat NGF probe revealed that the level of HDNF mRNA in kidney was only slightly

higher than the levels of NGF mRNA in peripheral sympathetic target tissues, indicating that HDNF is produced in relatively small amounts in peripheral rat tissues. This is also true for the brain, and the fact that seven positive cDNA clones were isolated from 1.2×10^6 independent clones suggests that in hippocampus, containing the highest level of NGF mRNA, this transcript constitutes ≈1 in every 1/0,000, which clearly represents a rare transcript. Thus, as in the case of NGF, HDNF may be present in limiting amounts and functions *in vivo* as a target-derived factor for a specific subset of both peripheral and central neurons. The regional distribution of HDNF mRNA in the periphery is, however, different from NGF, and, in agreement with the *in vitro* biological assays, HDNF may support a different set of peripheral neurons. Of interest is also that HDNF mRNA was found in the ovary, whereas no mRNA was detected in the testis, where both NGF and its receptor is expressed (48) and where NGF has been suggested to mediate an interaction between Sertoli cells and germ cells (49). This shows that different members of the NGF family are expressed in different reproductive tissues and suggests that they may have nonoverlapping functions outside the nervous system.

Interestingly, the three neurotrophic proteins were maximally expressed at different times of brain development with a peak of HDNF mRNA shortly after birth, BDNF mRNA around 2 weeks, and NGF mRNA around 3 weeks after birth (see ref. 8 for NGF). Moreover, the mRNA's for all three proteins were expressed in hippocampus at levels higher than in other regions, particularly in the case of HDNF. Within hippocampus, all three mRNAs were also confined to neurons (see ref. 10 for NGF) and a clear topographical division was seen, where HDNF mRNA was concentrated to pyramidal neurons in medial CA1, CA2, and granular neurons in dentate gyrus. Strongly labeled BDNF neurons were primarily seen in CA3 and the hilar region of dentate gyrus. Neurons with apparent lower levels of BDNF mRNA were seen in the dentate gyrus. The hilar region, containing neurons with high levels of BDNF mRNA, showed no labeling for HDNF mRNA.

This remarkable concentration of trophic factors in the adult hippocampus suggests that maintenance of plasticity is crucial to its function and may relate to the presumed morphological sequelae of long-term potentiation and memory consolidation processes. The intriguing temporal and spatial expression of the three neurotrophic proteins in the brain suggests that they predominantly support neuronal innervation at different times of development and that they may also exert specific trophic support for different central nervous system neurons, a possibility that will be an interesting topic for future studies.

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